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Derivation of a Theory to Estimate the
Kinetic Parameters for Systems with Multiple
Substrates Competing for the Same Active
Site on an Enzyme

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Abstract

In studies to characterize the kinetic parameters for catalytic hydrolysis of the organophosphorus nerve agent 1,2,2-trimethylpropyl methylphosphonofluoridate (GD) by human paraoxonase (HuPON1), it was found that standard analysis techniques could not be employed because the kinetic parameters for the GD isomers were not significantly different from each other. Separation of the racemic mixture into component stereoisomers for individual study is feasible but extremely expensive. It was recognized that a theory was required that would allow estimates of kinetic parameters of each isomer to be obtained under conditions of simultaneous hydrolysis of all four stereoisomers. This report presents a synopsis of the derivation of theoretical solutions for the time-course of the four substrates (the stereoisomers) undergoing simultaneous hydrolysis by a single active site of an enzyme. The enzyme “steady state” assumption made in the derivation is tested by comparing the theoretical solution against solutions obtained by high accuracy numerical integration of the state equations. The theoretical solutions are used to estimate the kinetic parameters for racemic GD hydrolysis by human PON1. The graphs of the theoretical solutions onto Lineweaver-Burke, Eadie-Hofstee, and Hanes-Woolf plots provide visual demonstrations of the inadequacy of those techniques for the determination of kinetic parameters for multiple substrate systems.

Introduction

The reaction system to be modeled consisted of four substrates (the stereoisomers of the nerve agent soman, GD) being simultaneously hydrolyzed by an enzyme (human paraoxonase) to produce four hydrolysis products as depicted in Figure 1 shown below, which describes the relationships among the concentrations of the components. Each substrate (A, B, C, D) combines with free enzyme (E) according to a bimolecular rate constant (k_1 , k_4 , k_7 , k_{10}) to form an enzyme-substrate intermediate (E_A , E_B , E_C , E_D) that progresses either forward according to a first order rate constant (k_3 , k_6 , k_9 , k_{12}) toward the hydrolyzed substrate (P, Q, R, S) and free enzyme or backward according to a first order rate constant (k_2 , k_5 , k_8 , k_{11}) to give substrate and free enzyme. The enzyme is assumed to initially start entirely as free enzyme with concentration E_0 . Conservation of mass dictates that $E_0 = E + E_A + E_B + E_C + E_D$ at any time.

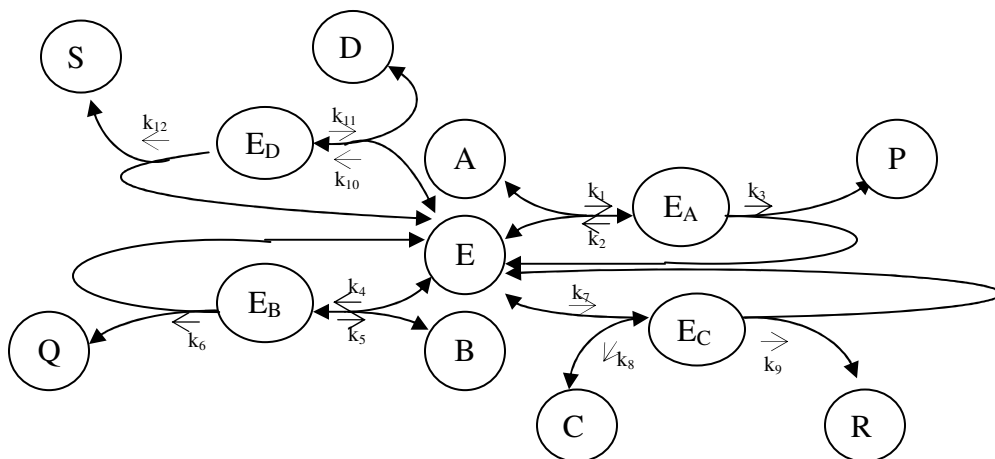


Figure 1. Reaction schematic of the racemic GD-HuPON system. The stereoisomers of GD are shown as A, B, C and D; the free form of HuPON as E; the GD-PON intermediates by E_A , E_B , E_C and E_D ; and the hydrolysis products by P, Q, R and S. Bimolecular and first-order rate constants are represented by the symbols k_1 through k_{12} .

Derivation of GD-PON Intermediate "Steady State" Levels

Mass balances on the enzyme-substrate complexes result in the state equations for the enzyme-substrate intermediates.

$$\begin{aligned} dE_A/dt &= k_1 (A) (E) - (k_2 + k_3) (E_A) \\ dE_B/dt &= k_4 (B) (E) - (k_5 + k_6) (E_B) \\ dE_C/dt &= k_7 (C) (E) - (k_8 + k_9) (E_C) \\ dE_D/dt &= k_{10} (D) (E) - (k_{11} + k_{12}) (E_D) \end{aligned}$$

After the enzyme "steady state" assumption (that the rates of change of the intermediates are zero after enzyme steady state has been achieved), the following relationships are obtained:

$$\begin{aligned} 0 &= k_1 (A) (E_0 - E_A - E_B - E_C - E_D) - (k_2 + k_3) (E_A) \\ 0 &= k_4 (B) (E_0 - E_A - E_B - E_C - E_D) - (k_5 + k_6) (E_B) \\ 0 &= k_7 (C) (E_0 - E_A - E_B - E_C - E_D) - (k_8 + k_9) (E_C) \\ 0 &= k_{10} (D) (E_0 - E_A - E_B - E_C - E_D) - (k_{11} + k_{12}) (E_D) \end{aligned}$$

where the free enzyme level has been written as $(E_0 - E_A - E_B - E_C - E_D)$. The equation set can be divided by E_0 and rearranged to write it in the form

$$\begin{aligned} 1 &= \{1 + (k_2 + k_3) / (k_1 A)\} E_A/E_0 + E_B/E_0 + E_C/E_0 + E_D/E_0 \\ 1 &= E_A/E_0 + \{1 + (k_5 + k_6) / (k_4 B)\} E_B/E_0 + E_C/E_0 + E_D/E_0 \\ 1 &= E_A/E_0 + E_B/E_0 + \{1 + (k_8 + k_9) / (k_7 C)\} E_C/E_0 + E_D/E_0 \\ 1 &= E_A/E_0 + E_B/E_0 + E_C/E_0 + \{1 + (k_{11} + k_{12}) / (k_{10} D)\} E_D/E_0 \end{aligned}$$

which may be expressed as the matrix equation

$$\begin{matrix} \rightarrow & & \rightarrow \\ \mathbf{U} & = & \mathbf{M} \mathbf{E} \\ \mathbf{4 \times 1} & & \mathbf{4 \times 4} \quad \mathbf{4 \times 1} \end{matrix} \quad \text{(equation 1)}$$

where $\mathbf{U} = (1, 1, 1, 1)^T$; $\mathbf{E} = (E_A/E_0, E_B/E_0, E_C/E_0, E_D/E_0)^T$; and the matrix \mathbf{M} is given by

$$\mathbf{M} = \begin{bmatrix} 1+a & 1 & 1 & 1 \\ 1 & 1+b & 1 & 1 \\ 1 & 1 & 1+c & 1 \\ 1 & 1 & 1 & 1+d \end{bmatrix}$$

where $a = (k_2 + k_3) / (k_1 A) = K_{mA}/A$; $b = (k_5 + k_6) / (k_4 B) = K_{mB}/B$; $c = (k_8 + k_9) / (k_7 C) = K_{mC}/C$; and $d = (k_{11} + k_{12}) / (k_{10} D) = K_{mD}/D$.

The inverse of \mathbf{M} is

$$\mathbf{M}^{-1} = \begin{bmatrix} bcd+bc+bd+cd & -(cd) & -bd & -(bc) \\ -(cd) & acd+ac+ad+cd & -(ad) & -ac \\ -bd & -(ad) & abd+ab+ad+bd & -(ab) \\ -(bc) & -ac & -(ab) & abc+ab+ac+bc \end{bmatrix}$$

$$abcd + bcd + acd + abd + abc$$

Pre-multiplying equation 1 by \mathbf{M}^{-1} results in a solution for the vector \mathbf{E} of steady state enzyme intermediate levels as functions of the substrate concentrations and the system parameters.

$$\begin{matrix} \rightarrow \\ \mathbf{E} \\ 4 \times 1 \end{matrix} = (bcd, acd, abd, abc)^T (abcd+bcd+acd+abd+abc)^{-1}$$

Replacing the symbols a, b, c, and d using their definitions, we express the fraction of enzyme existing in each intermediate form at enzyme steady state in terms of the system parameters

$$\begin{aligned} E_A/E_0 &= (A/K_{mA}) / (1 + A/K_{mA} + B/K_{mB} + C/K_{mC} + D/K_{mD}) \\ E_B/E_0 &= (B/K_{mB}) / (1 + A/K_{mA} + B/K_{mB} + C/K_{mC} + D/K_{mD}) \\ E_C/E_0 &= (C/K_{mC}) / (1 + A/K_{mA} + B/K_{mB} + C/K_{mC} + D/K_{mD}) \\ E_D/E_0 &= (D/K_{mD}) / (1 + A/K_{mA} + B/K_{mB} + C/K_{mC} + D/K_{mD}) \end{aligned}$$

The fraction of free enzyme existing at enzyme steady state is given by

$$E/E_0 = 1 / (1 + A/K_{mA} + B/K_{mB} + C/K_{mC} + D/K_{mD})$$

Rates of Substrate hydrolysis

We write the rates of change of the substrates at enzyme steady state

$$\begin{aligned} dA/dt &= -k_1 A E + k_2 E_A \\ dB/dt &= -k_4 B E + k_5 E_B \\ dC/dt &= -k_7 C E + k_8 E_C \\ dD/dt &= -k_{10} D E + k_{11} E_D \end{aligned}$$

and substitute the steady state enzyme levels. After rearrangement we find that

$$\begin{aligned} (dA/dt) / (k_2 E_0 A/K_{mA} - k_1 A E_0) &= (1 + A/K_{mA} + B/K_{mB} + C/K_{mC} + D/K_{mD})^{-1} \\ (dB/dt) / (k_5 E_0 B/K_{mB} - k_4 B E_0) &= (1 + A/K_{mA} + B/K_{mB} + C/K_{mC} + D/K_{mD})^{-1} \\ (dC/dt) / (k_8 E_0 C/K_{mC} - k_7 C E_0) &= (1 + A/K_{mA} + B/K_{mB} + C/K_{mC} + D/K_{mD})^{-1} \\ (dD/dt) / (k_{11} E_0 D/K_{mD} - k_{10} D E_0) &= (1 + A/K_{mA} + B/K_{mB} + C/K_{mC} + D/K_{mD})^{-1} \end{aligned}$$

(equations 2)

The right hand sides of the four equations are the same, so we may equate all pairs of the left hand sides to obtain 6 differential relationships between the substrates

$$\begin{aligned}
(dA/dt) / (k_2 E_0 A / K_{mA} - k_1 A E_0) &= (dB/dt) / (k_5 E_0 B / K_{mB} - k_4 B E_0) & (A \text{ \& } B) \\
(dA/dt) / (k_2 E_0 A / K_{mA} - k_1 A E_0) &= (dC/dt) / (k_8 E_0 C / K_{mC} - k_7 C E_0) & (A \text{ \& } C) \\
(dA/dt) / (k_2 E_0 A / K_{mA} - k_1 A E_0) &= (dD/dt) / (k_{11} E_0 D / K_{mD} - k_{10} D E_0) & (A \text{ \& } D) \\
(dB/dt) / (k_5 E_0 B / K_{mB} - k_4 B E_0) &= (dC/dt) / (k_8 E_0 C / K_{mC} - k_7 C E_0) & (B \text{ \& } C) \\
(dB/dt) / (k_5 E_0 B / K_{mB} - k_4 B E_0) &= (dD/dt) / (k_{11} E_0 D / K_{mD} - k_{10} D E_0) & (B \text{ \& } D) \\
(dC/dt) / (k_8 E_0 C / K_{mC} - k_7 C E_0) &= (dD/dt) / (k_{11} E_0 D / K_{mD} - k_{10} D E_0) & (C \text{ \& } D)
\end{aligned}$$

These relationships can be integrated with respect to time from $0 \rightarrow T$ to obtain relationships between the substrates at any time

$$\begin{aligned}
A/A_0 &= (B/B_0)^p ; B/B_0 = (A/A_0)^{1/p} & \text{where } p &= (K_{mB}/K_{mA}) (V_{maxA}/V_{maxB}) \\
A/A_0 &= (C/C_0)^q ; C/C_0 = (A/A_0)^{1/q} & \text{where } q &= (K_{mC}/K_{mA}) (V_{maxA}/V_{maxC}) \\
A/A_0 &= (D/D_0)^r ; D/D_0 = (A/A_0)^{1/r} & \text{where } r &= (K_{mD}/K_{mA}) (V_{maxA}/V_{maxD}) \\
B/B_0 &= (C/C_0)^s ; C/C_0 = (B/B_0)^{1/s} & \text{where } s &= (K_{mC}/K_{mB}) (V_{maxB}/V_{maxC}) \\
B/B_0 &= (D/D_0)^t ; D/D_0 = (B/B_0)^{1/t} & \text{where } t &= (K_{mD}/K_{mB}) (V_{maxB}/V_{maxD}) \\
C/C_0 &= (D/D_0)^u ; D/D_0 = (C/C_0)^{1/u} & \text{where } u &= (K_{mD}/K_{mC}) (V_{maxC}/V_{maxD})
\end{aligned}$$

And where $V_{maxA} = k_3 E_0$; $V_{maxB} = k_6 E_0$; $V_{maxC} = k_9 E_0$; $V_{maxD} = k_{12} E_0$

Using these relationships, we replace the variables in equations 2 in favor of each equation's substrate and rearrange the resulting equations to obtain

$$\begin{aligned}
-V_{maxA}/K_{mA} &= \left((A/A_0)^{-1} + A_0 K_{mA}^{-1} + C_0 (A/A_0)^{(1/q)-1} K_{mC}^{-1} + B_0 (A/A_0)^{(1/p)-1} K_{mB}^{-1} + D_0 (A/A_0)^{(1/r)-1} K_{mD}^{-1} \right) d/dt \{A/A_0\} \\
-V_{maxB}/K_{mB} &= \left((B/B_0)^{-1} + A_0 (B/B_0)^{p-1} K_{mA}^{-1} + C_0 (B/B_0)^{(1/s)-1} K_{mC}^{-1} + B_0 K_{mB}^{-1} + D_0 (B/B_0)^{(1/t)-1} K_{mD}^{-1} \right) d/dt \{B/B_0\} \\
-V_{maxC}/K_{mC} &= \left((C/C_0)^{-1} + A_0 (C/C_0)^{q-1} K_{mA}^{-1} + C_0 K_{mC}^{-1} + B_0 (C/C_0)^{s-1} K_{mB}^{-1} + D_0 (C/C_0)^{(1/u)-1} K_{mD}^{-1} \right) d/dt \{C/C_0\} \\
-V_{maxD}/K_{mD} &= \left((D/D_0)^{-1} + A_0 (D/D_0)^{r-1} K_{mA}^{-1} + C_0 (D/D_0)^{u-1} K_{mC}^{-1} + B_0 (D/D_0)^{t-1} K_{mB}^{-1} + D_0 K_{mD}^{-1} \right) d/dt \{D/D_0\}
\end{aligned}$$

Where

$$\begin{aligned}
p &= (K_{mB}/K_{mA}) (V_{maxA}/V_{maxB}) ; q = (K_{mC}/K_{mA}) (V_{maxA}/V_{maxC}) ; r = (K_{mD}/K_{mA}) (V_{maxA}/V_{maxD}) \\
s &= (K_{mC}/K_{mB}) (V_{maxB}/V_{maxC}) ; t = (K_{mD}/K_{mB}) (V_{maxB}/V_{maxD}) ; u = (K_{mD}/K_{mC}) (V_{maxC}/V_{maxD})
\end{aligned}$$

(equations 3)

The above equations each contain a single variable and are of the form

$$\text{constant} = F \{ x(t) \} d/dt \{ x(t) \},$$

which can be integrated with respect to time to obtain functions of the form, $\int F\{x\}dx = \text{constant} \cdot T$. Each integral can be evaluated on the interval 0 to T to obtain equations relating time to the fractional substrate at that time level.

For the first equation in equation set 3,

$$-V_{\max A}/K_{mA} = \left((A/A_0)^{-1} + A_0 K_{mA}^{-1} + B_0 (A/A_0)^{(1/p)-1} K_{mB}^{-1} + C_0 (A/A_0)^{(1/q)-1} K_{mC}^{-1} + D_0 (A/A_0)^{(1/r)-1} K_{mD}^{-1} \right) d/dt \{A/A_0\}$$

We consider each term separately.

$$\begin{aligned} -V_{\max A}/K_{mA} = & (A/A_0)^{-1} d/dt \{A/A_0\} + A_0 K_{mA}^{-1} d/dt \{A/A_0\} \\ & + B_0 (A/A_0)^{(1/p)-1} K_{mB}^{-1} d/dt \{A/A_0\} \\ & + C_0 (A/A_0)^{(1/q)-1} K_{mC}^{-1} d/dt \{A/A_0\} \\ & + D_0 (A/A_0)^{(1/r)-1} K_{mD}^{-1} d/dt \{A/A_0\} \end{aligned}$$

Integrate each term with respect to time from

$$\begin{aligned} \int -V_{\max A}/K_{mA} dt = & \int (A/A_0)^{-1} d/dt \{A/A_0\} dt + \int A_0 K_{mA}^{-1} d/dt \{A/A_0\} dt \\ & + \int B_0 K_{mB}^{-1} (A/A_0)^{(1/p)-1} d/dt \{A/A_0\} dt \\ & + \int C_0 K_{mC}^{-1} (A/A_0)^{(1/q)-1} d/dt \{A/A_0\} dt \\ & + \int D_0 K_{mD}^{-1} (A/A_0)^{(1/r)-1} d/dt \{A/A_0\} dt \end{aligned}$$

And evaluate at t=T and t=0

$$\begin{aligned} \left[-V_{\max A}/K_{mA} t \right] \Big|_{t=0}^{t=T} = & \left[\ln \{A<t>/A_0\} \right] \Big|_{t=0}^{t=T} + \left[A_0 K_{mA}^{-1} A<t>/A_0 \right] \Big|_{t=0}^{t=T} \\ & + \left[B_0 K_{mB}^{-1} (p) (A<t>/A_0)^{(1/p)} \right] \Big|_{t=0}^{t=T} \\ & + \left[C_0 K_{mC}^{-1} (q) (A<t>/A_0)^{(1/q)} \right] \Big|_{t=0}^{t=T} \\ & + \left[D_0 K_{mD}^{-1} (r) (A<t>/A_0)^{(1/r)} \right] \Big|_{t=0}^{t=T} \end{aligned}$$

to obtain

$$\begin{aligned} -V_{\max A}/K_{mA} T - 0 = & \ln \{A<T>/A_0\} - \ln \{A<0>/A_0\} \\ & + A_0 K_{mA}^{-1} A<T>/A_0 - A_0 K_{mA}^{-1} A<0>/A_0 \\ & + p B_0 K_{mB}^{-1} (A<T>/A_0)^{(1/p)} - p B_0 K_{mB}^{-1} (A<0>/A_0)^{(1/p)} \\ & + q C_0 K_{mC}^{-1} (A<T>/A_0)^{(1/q)} - q C_0 K_{mC}^{-1} (A<0>/A_0)^{(1/q)} \\ & + r D_0 K_{mD}^{-1} (A<T>/A_0)^{(1/r)} - r D_0 K_{mD}^{-1} (A<0>/A_0)^{(1/r)} \\ -V_{\max A}/K_{mA} T = & \ln \{A/A_0\} + A_0 K_{mA}^{-1} A/A_0 - A_0 K_{mA}^{-1} \\ & + p B_0 K_{mB}^{-1} (A/A_0)^{(1/p)} - B_0 K_{mB}^{-1} \\ & + q C_0 K_{mC}^{-1} (A/A_0)^{(1/q)} - C_0 K_{mC}^{-1} \\ & + r D_0 K_{mD}^{-1} (A/A_0)^{(1/r)} - D_0 K_{mD}^{-1} \end{aligned}$$

We replace p, q, and r using their definitions (but not in the exponents) to write

$$\begin{aligned}
-V_{\max A}/K_{mA} \ T &= \ln \{A/A_0\} + A_0 K_{mA}^{-1} \{ A/A_0 - 1 \} \\
&\quad + (K_{mB}/K_{mA}) (V_{\max A}/V_{\max B}) B_0 K_{mB}^{-1} \{ (A/A_0)^{(1/p)} - 1 \} \\
&\quad + (K_{mC}/K_{mA}) (V_{\max A}/V_{\max C}) C_0 K_{mC}^{-1} \{ (A/A_0)^{(1/q)} - 1 \} \\
&\quad + (K_{mD}/K_{mA}) (V_{\max A}/V_{\max D}) D_0 K_{mD}^{-1} \{ (A/A_0)^{(1/r)} - 1 \} \\
\\
T &= - (K_{mA}/V_{\max A}) \ln \{A/A_0\} \\
&\quad - A_0 K_{mA}^{-1} \{ A/A_0 - 1 \} \\
&\quad - (K_{mA}/V_{\max A}) (K_{mB}/K_{mA}) (V_{\max A}/V_{\max B}) B_0 K_{mB}^{-1} \{ (A/A_0)^{(1/p)} - 1 \} \\
&\quad - (K_{mA}/V_{\max A}) (K_{mC}/K_{mA}) (V_{\max A}/V_{\max C}) C_0 K_{mC}^{-1} \{ (A/A_0)^{(1/q)} - 1 \} \\
&\quad - (K_{mA}/V_{\max A}) (K_{mD}/K_{mA}) (V_{\max A}/V_{\max D}) D_0 K_{mD}^{-1} \{ (A/A_0)^{(1/r)} - 1 \}
\end{aligned}$$

From which we obtain

$$\begin{aligned}
T_A &= (A_0/V_{\max A}) (1 - (A/A_0)^{(K_{mA}/K_{mA}) (V_{\max A}/V_{\max A})}) \\
&\quad + (B_0/V_{\max B}) (1 - (A/A_0)^{(K_{mA}/K_{mB}) (V_{\max B}/V_{\max A})}) \\
&\quad + (C_0/V_{\max C}) (1 - (A/A_0)^{(K_{mA}/K_{mC}) (V_{\max C}/V_{\max A})}) \\
&\quad + (D_0/V_{\max D}) (1 - (A/A_0)^{(K_{mA}/K_{mD}) (V_{\max D}/V_{\max A})}) \\
&\quad - (K_{mA}/V_{\max A}) \ln (A/A_0)
\end{aligned}$$

Similarly, we obtain from the other three equations

$$\begin{aligned}
T_B &= + (A_0/V_{\max A}) (1 - (B/B_0)^{(K_{mB}/K_{mA}) (V_{\max A}/V_{\max B})}) \\
&\quad + (B_0/V_{\max B}) (1 - (B/B_0)^{(K_{mB}/K_{mB}) (V_{\max B}/V_{\max B})}) \\
&\quad + (C_0/V_{\max C}) (1 - (B/B_0)^{(K_{mB}/K_{mC}) (V_{\max C}/V_{\max B})}) \\
&\quad + (D_0/V_{\max D}) (1 - (B/B_0)^{(K_{mB}/K_{mD}) (V_{\max D}/V_{\max B})}) \\
&\quad - (K_{mB}/V_{\max B}) \ln (B/B_0)
\end{aligned}$$

$$\begin{aligned}
T_C &= (A_0/V_{\max A}) (1 - (C/C_0)^{(K_{mC}/K_{mA}) (V_{\max A}/V_{\max C})}) \\
&\quad + (B_0/V_{\max B}) (1 - (C/C_0)^{(K_{mC}/K_{mB}) (V_{\max B}/V_{\max C})}) \\
&\quad + (C_0/V_{\max C}) (1 - (C/C_0)^{(K_{mC}/K_{mC}) (V_{\max C}/V_{\max C})}) \\
&\quad + (D_0/V_{\max D}) (1 - (C/C_0)^{(K_{mC}/K_{mD}) (V_{\max D}/V_{\max C})}) \\
&\quad - (K_{mC}/V_{\max C}) \ln (C/C_0)
\end{aligned}$$

$$\begin{aligned}
T_D &= (A_0/V_{\max A}) (1 - (D/D_0)^{(K_{mD}/K_{mA}) (V_{\max A}/V_{\max D})}) \\
&\quad + (B_0/V_{\max B}) (1 - (D/D_0)^{(K_{mD}/K_{mB}) (V_{\max B}/V_{\max D})}) \\
&\quad + (C_0/V_{\max C}) (1 - (D/D_0)^{(K_{mD}/K_{mC}) (V_{\max C}/V_{\max D})}) \\
&\quad + (D_0/V_{\max D}) (1 - (D/D_0)^{(K_{mD}/K_{mD}) (V_{\max D}/V_{\max D})}) \\
&\quad - (K_{mD}/V_{\max D}) \ln (D/D_0)
\end{aligned}$$

(equations 4)

Although complex, these equations give the time it would take for each substrate (normalized by its initial level) to reach a particular level. As such, they can be used to form graphs of the substrate levels as functions of time by treating the fractional substrate level (X/X_0) as the independent variable. Since the enzyme intermediate levels start at zero, the fractional substrate levels can only fall during hydrolysis. Therefore, they each start at a value of one and fall towards a value of zero (most likely at different rates) as the substrates are hydrolyzed.

Application of the Theory

In studies where racemic GD was hydrolyzed by human PON1, the activity of each GD isomer was measured at various times during the hydrolysis (as detailed in “Direct detection of stereospecific soman (GD) hydrolysis by human serum paraoxonase [HuPON1]” by Yeung et al., FEBS Journal, in press). The functions giving the theoretical solutions for the four isomers (equation set 4) were plotted on an X-Y chart with the fractional substrate levels on the ordinate and the time taken for the substrate to fall to a particular substrate level on the abscissa. Measured stereoisomer concentrations and times were also plotted on the chart. The kinetic parameters of the equations were adjusted to fit the theoretical curves to the actual data. The SigmaPlot® and Prism® nonlinear regression curve fitting programs were used in an attempt to automate the curve fitting process, but the methods could not be made to converge, presumably because of the large number of parameters (12) and the complexity of the functions. Consequently, the curves were fit manually by adjusting parameters in an Excel® workbook that had been programmed to compute and chart the theoretical solutions along with measured data points.

A standard assumption when dealing with enzyme-substrate systems is that the maximum rate of product generation (and substrate consumption) occurs at the beginning of the enzyme action. While this is true in a single substrate system, in multiple substrate systems the hydrolysis of a particular isomer can start slowly because the enzyme is busy hydrolyzing another substrate. The hydrolysis of the substrate can speed up when the enzyme becomes available. With this concept in mind, the V_{\max} value for an isomer was set to the maximum hydrolysis velocity measured at any time during the hydrolysis experiment. Given these V_{\max} values and the known starting enzyme concentration, E_0 , estimates of the kinetic parameters (k_3 , k_6 , k_9 , k_{12}) were found. Under the assumption that the single enzyme intermediate could disassociate in the forward or reverse directions with equal facility, the parameters (k_2 , k_5 , k_8 , k_{11}) were set equal to the values for (k_3 , k_6 , k_9 , k_{12}). This left just the bimolecular rate constants (k_1 , k_4 , k_7 , k_{10}) to be adjusted using curve fitting. The results for a particular initial GD level are shown below in Figure 2.

Hydrolysis of 0.37 mM racemic GD by Human PON1

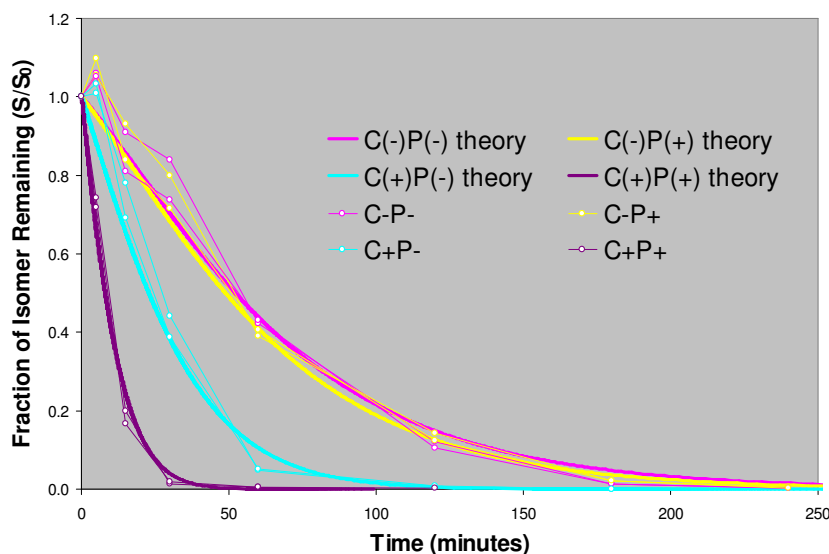


Figure 2. Curve Fitting of the Theoretical Solutions to Measured Data

Comparison with Numerical Simulations – Does the theory work?

To test the theoretical results, AcsIXtreme (a numerical simulation software package) was used to provide high accuracy numerical simulations of the original system. The results of the simulations were plotted on the X-Y Chart shown in Figure 3.

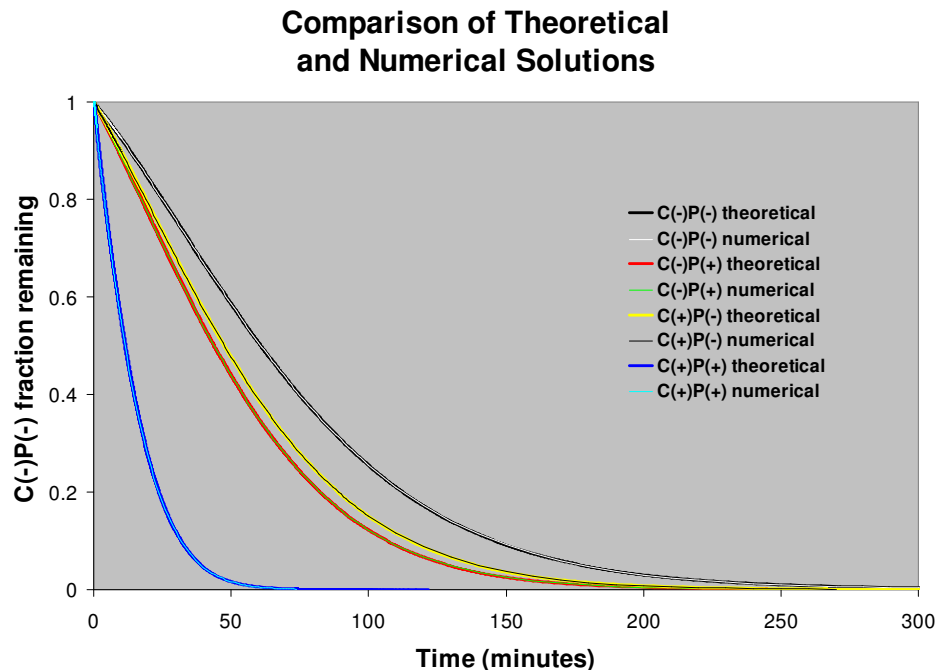


Figure 3. The theoretical solutions for the four substrates (drawn in bold) are shown with the numerical solutions. The solutions are identical.

To explore the amount of error introduced by the enzyme steady state assumption, the numerical levels of the enzyme intermediates were plotted along with the theoretical values for them predicted from the enzyme steady state assumption. The chart is shown in Figure 4.

Comparison of Assumed Enzyme "Steady State" Levels and Actual (Numerically Integrated) Levels

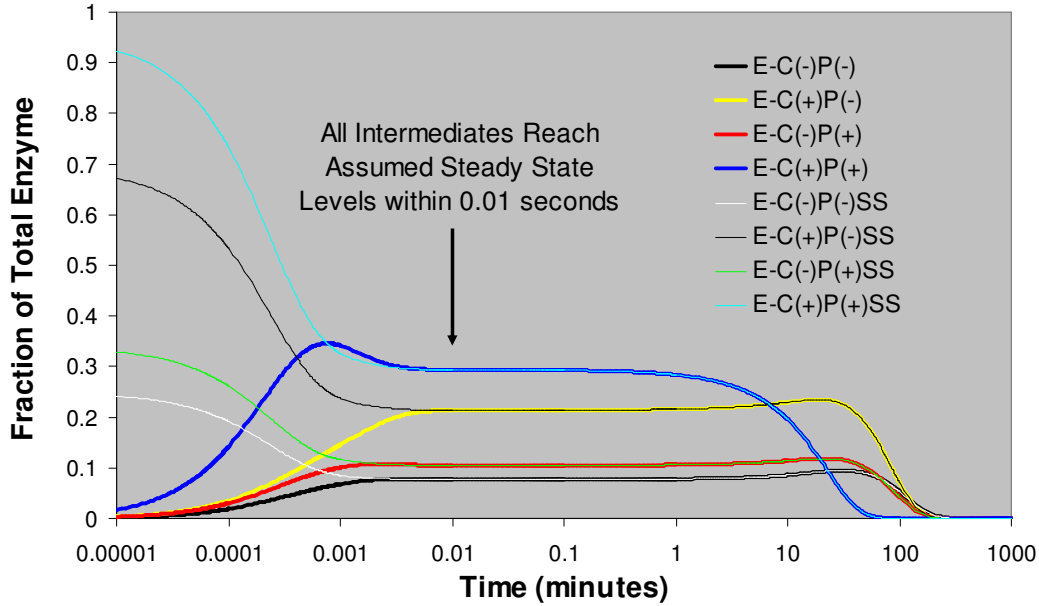


Figure 4. The effect of the enzyme steady state assumption. Note that the differences between the assumed and actual levels vanish quickly.

What about the single-substrate techniques?

It is illustrative to examine what the theoretical solutions would look like on Lineweaver-Burke, Eadie-Hofstee, and Hanes-Woolf plots. To accomplish this, we used the negative reciprocal of the rate of change of a substrate as the "1/Velocity" variable. The negative is used because the rate of the hydrolysis is the negative of the rate that the substrate changes. The reciprocals of the equations describing the rates of change of the substrates were rearranged and the relationships between the substrates were used to write each equation in terms of only a single substrate.

$$\begin{aligned}
 1/V_A = 1/V_{\max A} &+ (K_{mA}/V_{\max A}) (1/A) \\
 &+ (K_{mA}/V_{\max A} A_0) (B_0/K_{mB}) (A/A_0) (K_{mA}V_{\max B}/(K_{mB}V_{\max A}) - 1) \\
 &+ (K_{mA}/V_{\max A} A_0) (C_0/K_{mC}) (A/A_0) (K_{mA}V_{\max C}/(K_{mC}V_{\max A}) - 1) \\
 &+ (K_{mA}/V_{\max A} A_0) (D_0/K_{mD}) (A/A_0) (K_{mA}V_{\max D}/(K_{mD}V_{\max A}) - 1)
 \end{aligned}$$

$$\begin{aligned}
 1/V_B = 1/V_{\max B} &+ (K_{mB}/V_{\max B}) (1/B) \\
 &+ (K_{mB}/V_{\max B} B_0) (A_0/K_{mA}) (B/B_0) (K_{mB}V_{\max A}/(K_{mA}V_{\max B}) - 1) \\
 &+ (K_{mB}/V_{\max B} B_0) (C_0/K_{mC}) (B/B_0) (K_{mB}V_{\max C}/(K_{mC}V_{\max B}) - 1) \\
 &+ (K_{mB}/V_{\max B} B_0) (D_0/K_{mD}) (B/B_0) (K_{mB}V_{\max D}/(K_{mD}V_{\max B}) - 1)
 \end{aligned}$$

$$\begin{aligned}
 1/V_C = 1/V_{\max C} &+ (K_{mC}/V_{\max C}) (1/C) \\
 &+ (K_{mC}/V_{\max C} C_0) (A_0/K_{mA}) (C/C_0) (K_{mC}V_{\max A}/(K_{mA}V_{\max C}) - 1) \\
 &+ (K_{mC}/V_{\max C} C_0) (B_0/K_{mB}) (C/C_0) (K_{mC}V_{\max B}/(K_{mB}V_{\max C}) - 1)
 \end{aligned}$$

$$+ (K_{mC}/V_{maxC}C_0) (D_0/K_{mD}) (C/C_0) (K_{mC}V_{maxD}/(K_{mD}V_{maxC}) - 1)$$

$$\begin{aligned} 1/V_D = 1/V_{maxD} &+ (K_{mD}/V_{maxD}) (1/D) \\ &+ (K_{mD}/V_{maxD}D_0) (A_0/K_{mA}) (D/D_0) (K_{mD}V_{maxA}/(K_{mA}V_{maxD}) - 1) \\ &+ (K_{mD}/V_{maxD}D_0) (B_0/K_{mB}) (D/D_0) (K_{mD}V_{maxB}/(K_{mB}V_{maxD}) - 1) \\ &+ (K_{mD}/V_{maxD}D_0) (C_0/K_{mC}) (D/D_0) (K_{mD}V_{maxC}/(K_{mC}V_{maxD}) - 1) \end{aligned}$$

(equations 5)

The first two terms on the right of each equation give the standard (linear) L-B equation as it would apply to a single substrate. The last three terms are power functions of the fractional substrate levels. The terms add non-linear contributions to the (1/V) dimension. Figure 5 presents some measured data and the corresponding theoretical solutions plotted on the double reciprocal plot.

Lineweaver-Burke Plot of Theoretical Solutions and Measured Data for Hydrolysis of 1.67 mM Racemic GD by Human PON1

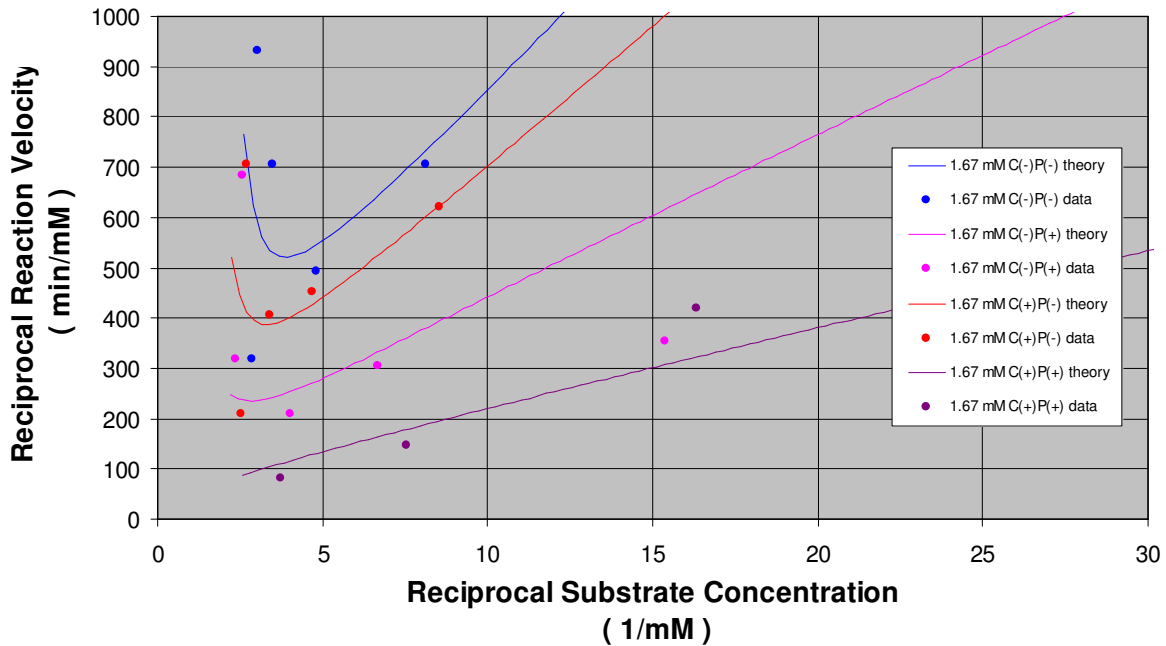


Figure 5. The Lineweaver-Burke plot for theoretical solutions of multi-substrate hydrolysis plotted along with measured data points. Note the nonlinearity for the higher substrate concentrations.

On this chart, each isomer starts at its highest (initial) concentration that occurs at the leftmost end of its curve. As the isomer level falls, the curve is traversed from left to right. It can be seen from the figure that the nonlinearity is most pronounced when the isomer concentrations are

highest. Note that the reaction velocities of three of the four isomers speed up (falling to lower $1/V$ levels) initially as the bulk of the enzyme, which was initially complexed with the fourth isomer, becomes available. Since the L-B transformation does not linearize the system, it is not appropriate to use the method to estimate the kinetics of a multiple substrate system.

Multiplying each equation in the equations 5 set by the corresponding substrate level gives the theoretical solutions in the Hanes-Woolf format.

$$\begin{aligned} \mathbf{A/V_A} = & A/V_{\max A} + (K_{mA}/V_{\max A}) + (K_{mA}/V_{\max A}) (B_0/K_{mB}) (A/A_0) (K_{mA}V_{\max B}) / (K_{mB}V_{\max A}) \\ & + (K_{mA}/V_{\max A}) (C_0/K_{mC}) (A/A_0) (K_{mA}V_{\max C}) / (K_{mC}V_{\max A}) \\ & + (K_{mA}/V_{\max A}) (D_0/K_{mD}) (A/A_0) (K_{mA}V_{\max D}) / (K_{mD}V_{\max A}) \end{aligned}$$

$$\begin{aligned} \mathbf{B/V_B} = & B/V_{\max B} + (K_{mB}/V_{\max B}) + (K_{mB}/V_{\max B}) (A_0/K_{mA}) (B/B_0) (K_{mB}V_{\max A}) / (K_{mA}V_{\max B}) \\ & + (K_{mB}/V_{\max B}) (C_0/K_{mC}) (B/B_0) (K_{mB}V_{\max C}) / (K_{mC}V_{\max B}) \\ & + (K_{mB}/V_{\max B}) (D_0/K_{mD}) (B/B_0) (K_{mB}V_{\max D}) / (K_{mD}V_{\max B}) \end{aligned}$$

$$\begin{aligned} \mathbf{C/V_C} = & C/V_{\max C} + (K_{mC}/V_{\max C}) + (K_{mC}/V_{\max C}) (A_0/K_{mA}) (C/C_0) (K_{mC}V_{\max A}) / (K_{mA}V_{\max C}) \\ & + (K_{mC}/V_{\max C}) (B_0/K_{mB}) (C/C_0) (K_{mC}V_{\max B}) / (K_{mB}V_{\max C}) \\ & + (K_{mC}/V_{\max C}) (D_0/K_{mD}) (C/C_0) (K_{mC}V_{\max D}) / (K_{mD}V_{\max C}) \end{aligned}$$

$$\begin{aligned} \mathbf{D/V_D} = & D/V_{\max D} + (K_{mD}/V_{\max D}) + (K_{mD}/V_{\max D}) (A_0/K_{mA}) (D/D_0) (K_{mD}V_{\max A}) / (K_{mA}V_{\max D}) \\ & + (K_{mD}/V_{\max D}) (B_0/K_{mB}) (D/D_0) (K_{mD}V_{\max B}) / (K_{mB}V_{\max D}) \\ & + (K_{mD}/V_{\max D}) (C_0/K_{mC}) (D/D_0) (K_{mD}V_{\max C}) / (K_{mC}V_{\max D}) \end{aligned}$$

(equations 6)

The first two terms in each equation give the single substrate form of the equations while the last three terms add nonlinearities to the S/V_S dimension.

Hanes-Woolf Plot of Theoretical Solutions and Measured Data for Hydrolysis of 1.67 mM Racemic GD by Human PON1

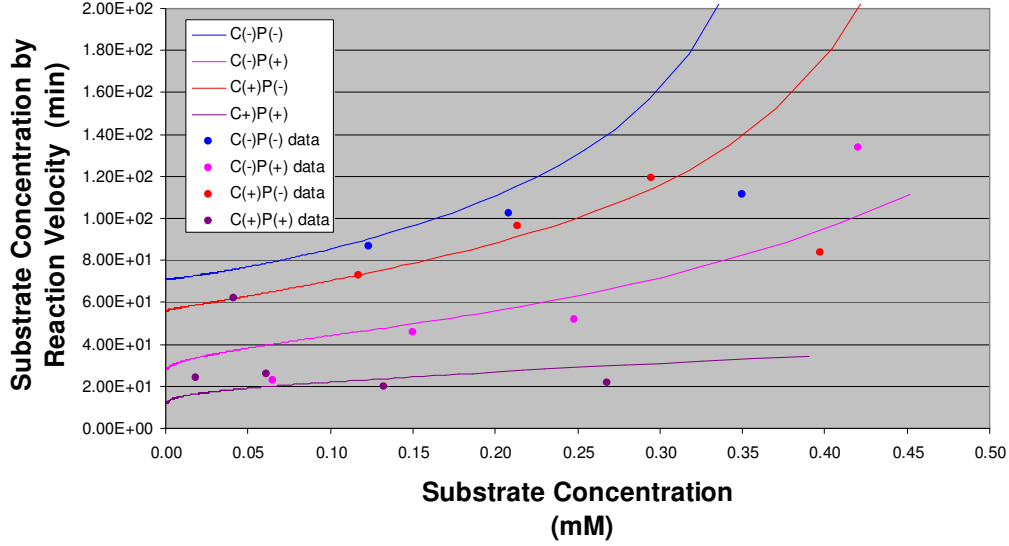


Figure 6. Hanes-Woolf plot of the multiple-substrate theoretical solutions and some measured data points.

Each equation in equations 5 may be multiplied by $V_S V_{\max S}$ and rearranged to form relationships that can be plotted on an Eadie-Hofstee plot.

$$V_A = V_{\max A} - K_{m A} \left\{ 1 + \left(B_0 / K_{m B} \right) (A / A_0) \frac{K_{m A} V_{\max B} / K_{m B} V_{\max A}}{K_{m A} V_{\max C} / K_{m C} V_{\max A}} + \left(C_0 / K_{m C} \right) (A / A_0) \frac{K_{m A} V_{\max D} / K_{m D} V_{\max A}}{K_{m A} V_{\max C} / K_{m C} V_{\max A}} \right\} (V_A / A)$$

$$V_B = V_{\max B} - K_{m B} \left\{ 1 + \left(A_0 / K_{m A} \right) (B / B_0) \frac{K_{m B} V_{\max A} / K_{m A} V_{\max B}}{K_{m B} V_{\max C} / K_{m C} V_{\max B}} + \left(C_0 / K_{m C} \right) (B / B_0) \frac{K_{m B} V_{\max D} / K_{m D} V_{\max B}}{K_{m B} V_{\max C} / K_{m C} V_{\max B}} \right\} (V_B / B)$$

$$V_C = V_{\max C} - K_{m C} \left\{ 1 + \left(A_0 / K_{m A} \right) (C / C_0) \frac{K_{m C} V_{\max A} / K_{m A} V_{\max C}}{K_{m C} V_{\max B} / K_{m B} V_{\max C}} + \left(B_0 / K_{m B} \right) (C / C_0) \frac{K_{m C} V_{\max D} / K_{m D} V_{\max C}}{K_{m C} V_{\max B} / K_{m B} V_{\max C}} \right\} (V_C / C)$$

$$V_D = V_{\max D} - K_{m D} \left\{ 1 + \left(A_0 / K_{m A} \right) (D / D_0) \frac{K_{m D} V_{\max A} / K_{m A} V_{\max D}}{K_{m D} V_{\max B} / K_{m B} V_{\max D}} + \left(B_0 / K_{m B} \right) (D / D_0) \frac{K_{m D} V_{\max C} / K_{m C} V_{\max D}}{K_{m D} V_{\max B} / K_{m B} V_{\max D}} \right\} (V_D / D)$$

(equations 7)

Eadie-Hofstee Plot of Theoretical Solutions and Measured Data for Hydrolysis of 1.67 mM Racemic GD by Human PON1

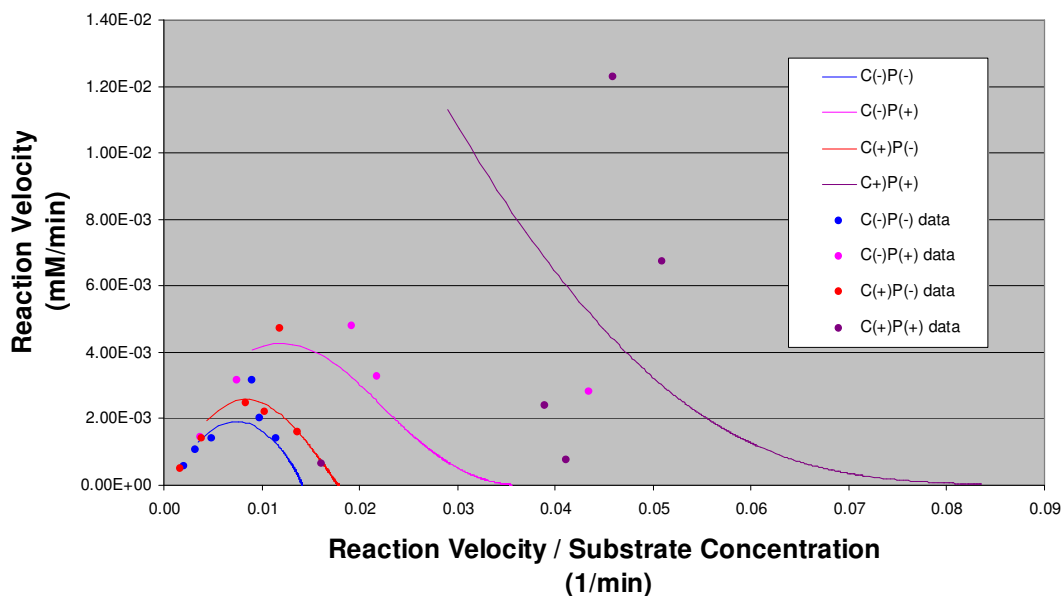


Figure 7. The data and theoretical solutions used in the previous two figures plotted on the Eadie-Hofstee plot. Single substrate systems plot as a straight line with slope = $-K_M$ and y-intercept = V_{max} .

Exploring the form of equations 7 provides an insight into the effect that substrate multiplicity has on the apparent affinity (K_M). The quantities in braces " $\{ \}$ " in the equations collapse to the value 1 when the initial levels of competing substrates are set to zero. The equations become the Eadie-Hofstee equations for independent single substrate systems. The effect of substrate multiplicity is to *increase* the apparent affinity that would be measured for a single substrate.

Conclusions

We conclude that the equations presented as equation set 4 are the correct theoretical solutions for the enzyme-substrate system presented as Figure 1 based upon the derivations and a comparison of numerical and theoretical solutions. The enzyme steady state assumption made in the derivation of the solutions has little, if any, effect on the solutions after an initial period less than one second. We have found that use of the theoretical solutions to estimate the kinetic parameters of the system is possible and yields sensible results. We can not conclude that the system presented as Figure 1 is an accurate representation of the GD-PON hydrolytic system although it does represent the simplest GD-PON system. The equations presented here are for a four substrate system, but systems of two and three substrates have also been solved and have solutions that are subsets of the presented work. A formal approach can be used to extend the equations to any number of multiple substrates. In general, this work should be useful for estimating the kinetic parameters of any multiple-substrate, single active site system. By setting kinetic parameters judiciously, this work should also prove useful in the study of enzyme inhibitors (organophosphates) and protectants (carbamates) that can both be viewed as special types of competitive substrates.